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Identification and endothelin-induced activation of multiple extracellular signal-regulated kinases in aortic smooth muscle cells

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In order to explore intracellular signaling pathways of the mitogenic action of endothelin (ET), we examined the effect of ET on activities of extracellular signal-regulated kinases (ERKs) in rat aortic smooth muscle cells (SMCs). Treatment of rat aortic SMCs with ET-1 increased kinase activities toward myelin basic protein (MBP). Both 43- and 41-kDa proteins were activated when kinase assays were done in MBP-containing polyacrylamide gels after SDS-PAGE. These proteins were identified as ERK1 and ERK2 with immunoprecipitation and immunoblotting using antipeptide antibodies, respectively. These results indicate that ERKs mediate signal transduction by ET.

Endothelin; Endothelin receptor; Aortic smooth muscle cell; Extracellular signal-regulated kinase; MBP/MAP2 kinase; Cell proliferation

1. INTRODUCTION

Endothelin (ET) was initially identified as a potent vasoconstrictor peptide produced by porcine vascular endothelial cells [1]. Analysis of the human ET gene revealed that ETs comprise a peptide family consisting of three isopeptides, ET-1, ET-2 and ET-3, which have subsequently been shown to have various effects on both vascular and non-vascular tissues [2,3]. Pharmacological studies and binding experiments have suggested the existence of multiple ET receptor (ETR) subtypes [4–6]. Subsequent molecular cloning studies have proved the existence of at least two ETR subtypes, ET-1-selective receptor and non-isopeptide-selective receptor, designated ET_AR and ET_BR, respectively [7,8]. Northern blot analysis of vascular tissues and cells with the cloned receptor cDNA probes showed that the major subtype of ETR in vascular smooth muscle cells (SMCs) is ET_AR [9,10], while the ET_BR subtype is predominantly expressed in vascular endothelial cells [11].

ET has been shown to stimulate DNA synthesis and increase cell numbers of cultured vascular SMCs [12–15]. ETR is coupled to phospholipase C, and ETR stimulation leads to activation of protein kinase C in many systems, including cultured vascular SMCs [16]. The mitogenic effect of ET has been demonstrated to be dependent, in part, on protein kinase C, because the promoting effect of ET on DNA synthesis was attenuated but not abolished in phorbol ester-pretreated, protein kinase C-depleted cells [14,15]. However, little is known about either the signal transduction mechanism acting downstream of protein kinase C or the protein kinase C-independent mitogenic signaling pathways.

Microtubule-associated protein 2 (MAP2) kinase or mitogen-activated protein (MAP) kinase, or myelin basic protein (MBP)/MAP2 kinases is/are rapidly stimulated in response to a variety of growth factors and other extracellular signals [17–27]. Their activation is accompanied by their phosphorylation on tyrosine, as well as serine/threonine residues, indicating their regulatory roles in a growth factor-dependent protein kinase cascade [18,20]. Recently, Boulton et al. reported the cDNA cloning of two related enzymes of extracellular signal-regulated kinases (KRK1 and ERK2, respectively) which are thought to encode MBP/MAP2 kinases [27,28], and the third related kinase, ERK3 [29].

In the present study we have identified two ERK isozymes, ERK1 and ERK2, in rat aortic SMCs, using two antibodies for immunoprecipitation and immunoblotting, one raised against the synthetic peptide corresponding to the C-terminal portion of the deduced amino acid sequence of the ERK1 and the other against the peptide containing the putative autophosphorylation sites of the amino acid sequence deduced from the ERK2 cDNA. We have also examined the effects of ET on activities of ERKs in rat aortic SMCs in order to elucidate the possible participation of ERKs in intracellular signaling pathways of ETRs.

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Abbreviations: ET, endothelin; ETR, endothelin receptor; SMC, smooth muscle cell; MAP2, microtubule-associated protein 2; MAP, mitogen-activated protein; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis and production of antisera*

The peptide, ELIFQETARFQPGAPEAP (peptide 1C), and the peptide, RIEVEQ ALAHPYLEQYYDPSDEP (peptide 2Y), which correspond to residues 350–367 and 299–321 of the amino acid sequence deduced from the ERK1 and ERK2 cDNA, respectively, were synthesized according to the solid-phase synthesis technique. The peptides (3 mg) were conjugated to bovine thyroglobulin (Sigma) (15.0 mg) using the carbodiimide coupling procedure [30]. The conjugate was emulsified with complete Freund's adjuvant and injected subcutaneously into multiple sites of female New Zealand White rabbits. Rabbit serum was collected after a number of booster injections. The antisera against peptide 1C and peptide 2Y were termed 1C antiserum and 2Y antiserum, respectively.

2.2. *Cell culture and preparation of cell extracts*

Rat aortic SMCs were prepared from explants of thoracic aorta of 20-week-old male Wistar rats and cultured according to the method previously described [31]. SMCs between the seventh and ninth passages were used. Chinese hamster ovary (CHO) cells were grown in α-minimum essential medium (Gibco) supplemented with 10% fetal calf serum. Cells were rendered quiescent by placing them in serum-free medium for 48 h. After treatment of the quiescent rat aortic SMCs with various concentrations of ET isopeptides (Peptide Institute, Inc., Minoh, Japan) for the indicated times, cells were lysed with 0.6 ml of lysis buffer containing 25 mM Tris-HCl (pH 7.6), 20 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 0.5 mM EGTA and 0.1 mM PMSF.

2.3. *Kinase assays*

Kinase assays toward MBP were performed at 30°C for 20 min in a final volume of 50 μl containing 10 mM $MgCl_2$, 40 μM ATP, 4 μM protein kinase inhibitor peptide (rabbit sequence) (Sigma), MBP (Sigma) (400 μg/ml), 1 μCi of [γ-^{32}P]ATP (Amersham Corp.) and the cell extract (30 μl). The reaction was stopped by adding 50 μl of 2 × Laemmli's sample buffer, and the samples were subjected to SDS-PAGE and autoradiography. The bands corresponding to the substrate were excised from the dried gels and the radioactivity was determined using a scintillation counter.

Kinase assays in MBP-containing polyacrylamide gels were carried out according to the method of Kameshita and Fujisawa [32]. The cell extracts were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg/ml MBP. SDS was removed from the gel by washing the gel with two changes of 25 ml each of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 h and then 250 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol for 1 h at room temperature. The enzyme was denatured by treating the gel first with two changes of 25 ml of 6 M guanidine-HCl at room temperature for 1 h and then renatured with four changes of 250 ml each of 50 mM Tris-HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol at 4°C. After renaturation, the gel was preincubated at 30°C for 30 min with 25 ml of 40 mM Tris-HCl (pH 8.0) containing 2 mM dithiothreitol and 10 mM $MgCl_2$. The gel was incubated at 30°C for 1 h with 28 ml of 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 50 μM ATP, and 10 μCi of [γ-^{32}]ATP. After incubation, the gel was washed with a 5% (w/v) trichloroacetic acid solution containing 1% pyrophosphate until the radioactivity of the solution became negligible. The washed gel was dried and then subjected to autoradiography.

2.4. *Immunoprecipitation and Western blotting*

The cell lysate was subjected to immunoprecipitation with 1C antiserum diluted 20-fold, and incubated for 1 h at 4°C after which 50 μl of protein A–agarose (Pharmacia) was added. After 2 h of incubation at 4°C, the immunoprecipitates were washed twice with lysis buffer. The kinase activity of the immunoprecipitates was assayed in MBP-containing polyacrylamide gels as described above. The cell lysate was subjected to SDS-PAGE and transferred to nitrocellulose sheets. They were incubated with each antibody, and proteins immunoreacting with antisera were detected using [^{125}I]protein A (Amersham Corp.).

3. RESULTS AND DISCUSSION

3.1. *Characterization of antisera*

Antisera were generated to two peptides predicted by the ERK1 and ERK2 cDNA. The specificity of antisera was determined by immunoblotting as shown in Fig. 1A. When cell extracts from CHO cells were blotted with antisera, one antibody against the C-terminal peptide of ERK1 recognized a 43-kDa band almost specifically (Fig. 1A, lane a). The other antibody raised against the ERK2 peptide, containing a sequence which resembles the regulatory autophosphorylated region of the insulin receptor, recognized 41- and 43-kDa proteins, although it probed the 41-kDa protein more efficiently (Fig. 1A, lane c). Recognition of the bands at 43 and 41 kDa was significantly blocked by preincubation of the antibodies with peptide antigens, demonstrating the specificity of the interaction of the antibodies with these proteins (Fig. 1A, lanes b and d). We conclude that the 43- and 41-kDa proteins correspond to ERK1 and ERK2, respectively, based on the relative specificity of the antisera raised against ERK1 and ERK2 peptides. This result is consistent with that of Boulton et al. who showed that the ERK1 and ERK2 gene products are highly related to the 43- and 41-kDa MBP/MAP2 kinases, respectively, using antibodies against ERK1 peptides and recombinant ERK2 protein [33]. Since 2Y antiserum was unable to immunoprecipitate significant amounts of the 43- and 41-kDa proteins under non-denaturing conditions, in the following experiments immunoprecipitation was done with the 1C antiserum, which is specific for the 43-kDa protein.

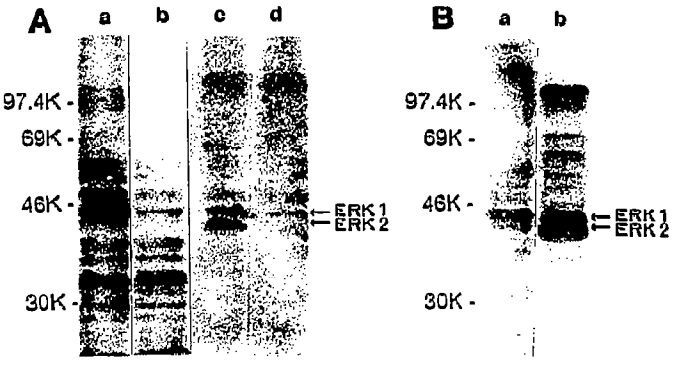

Fig. 1. Western blotting with antipeptide antibodies against ERKs. (A) Specificity of the antipeptide antibodies. Cell lysates from CHO cells were subjected to SDS-PAGE followed by Western blotting with antisera 1C (lane a) and 2Y (lane c) or the antibodies which had been preincubated with each of 500 μM peptides (lanes b and d). (B) Western blotting of cell lysates from rat aortic SMCs. The lysates were subjected to SDS-PAGE followed by immunoblotting with antisera 1C (lane a) and 2Y (lane b).

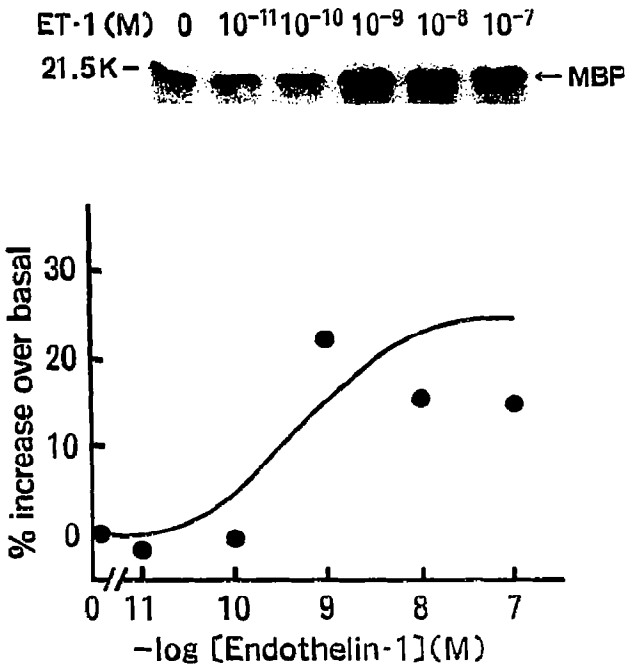

Fig. 2. Dose–response curve of ET-1 stimulation of kinase activity toward MBP in the cell lysate of rat aortic SMCs. Rat aortic SMCs were treated with the indicated concentrations of ET-1 for 5 min at 37°C. The cells were lysed and the cell lysate was subjected to kinase assays toward MBP. The bands were excised and counted by Cerenkov counting.

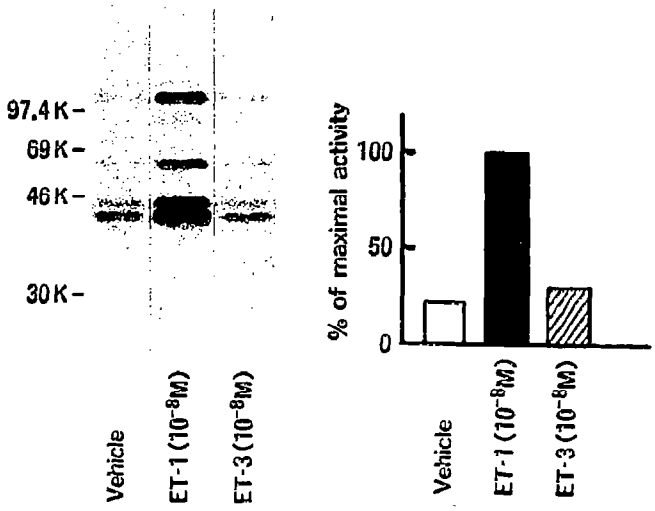

Fig. 3. Kinase assays of the cell lysate from rat aortic SMCs in MBP-containing gels after SDS-PAGE. The rat aortic SMCs were treated either without or with 10^{-8} M ET-1 or 10^{-8} M ET-3 for 5 min and lysed. The lysates were electrophoresed on SDS-polyacrylamide gels containing MBP. SDS was removed from the gel, and after denaturation with 6 M guanidine-HCl and renaturation in buffer containing 0.04% Tween 40, the gel was incubated with [γ-^{32}P]ATP and Mg^{2+} as described in Materials and Methods. The MBP kinase activity migrating at 41 kDa was measured by densitometric scanning of the autoradiogram.

3.2. *Identification of multiple ERKs in rat aortic SMCs*

The cell lysates from rat aortic SMCs were immunoblotted with the two antisera; 1C antiserum, which has selectivity for ERK1, and 2Y antiserum, which recognizes both ERK1 and ERK2 with a higher affinity for ERK2. As shown in Fig. 1B, 1C antiserum recognized a band at 43 kDa, while 2Y antiserum recognized a band at 41 kDa, and a band at 43 kDa with less intensity, identifing ERK isozymes in rat aortic SMCs (Fig. 1B, lanes a and b).

3.3. *Activation of kinase activity toward MBP by ET in rat aortic SMCs*

Treatment of rat aortic SMCs with ET-1 increased kinase activity toward MBP. The activation was dependent on the ET-1 concentration, as shown in Fig. 2. The concentration required for half-maximal activation was 5×10^{-10} ET-1. ET-3 at a concentration of 10^{-8} M did not increase kinase activity toward MBP (data not shown). This result suggests that ET-induced activation of kinase activity toward MBP may be mediated via ET_AR with selectivity for ET-1, which is predominantly expressed in vascular SMCs.

Maximal activation was almost attained 2–5 min after the addition of 10^{-8} M ET-1 to rat aortic SMCs. The activated kinase activity was then reduced to nearly basal levels within 30–60 min (data not shown).

3.4. *Identification of ET-1-stimulated kinase activity in rat aortic SMCs as ERKs*

To directly determine the molecular masses of the proteins responsible for the ET-1-stimulated kinase activity toward MBP, kinase assays in MBP-containing polyacrylamide gels after SDS-PAGE were done. The cell lysates of ET-1- and ET-3-treated or untreated cells were electrophoresed on an SDS-polyacrylamide gel containing MBP. As shown in Fig. 3, increased MBP kinase activities migrating at 43 and 41 kDa were observed after ET-1 treatment but not following ET-3 treatment, the molecular masses of which are consistent with those of ERK1 and ERK2, respectively. Both proteins were stimulated by ET-1 in a coordinate fashion. The MBP kinase activities, having molecular masses of approximately 100 kDa and 64 kDa, appeared to be stimulated by ET-1. Since the deduced amino acid sequence of the ERK3 predicts a protein of 63 kDa [29], the 64-kDa MBP kinase activity which apparently was stimulated by ET-1 in rat aortic SMCs may be closely related to ERK3. However, additional study is required to identify these MBP kinase activities.

When cell lysates from ET-1- and ET-3-treated or untreated cells were subjected to immunoprecipitation with 1C antiserum and the kinase assay of immunoprecipitates was done in MBP-containing gels, the MBP kinase activity migrating at 43 kDa was stimulated by ET-1 (Fig. 4). These results, coupled with those of kinase assays of cell lysates in MBP-containing gels, have

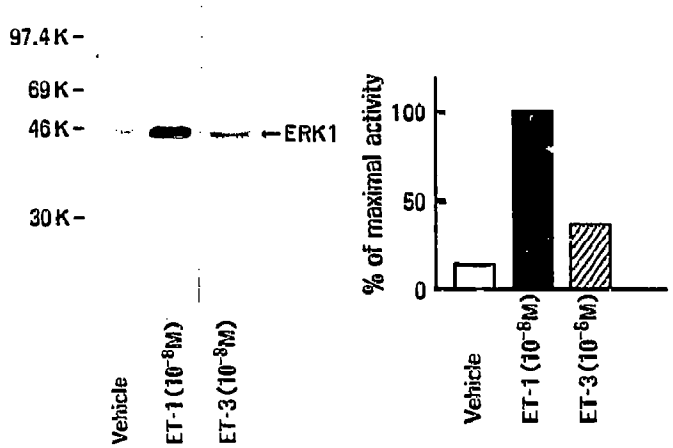

Fig. 4. Kinase assays of immunoprecipitates with the 1C antiserum in MBP-containing gels after SDS-PAGE. Rat aortic SMCs were treated either without or with 10^{-8} M ET-1 or 10^{-8} M ET-3 for 5 min. The cells were lysed and immunoprecipitated with 1C antiserum. The kinase assays of immunoprecipitates were done as described in Materials and Methods. The MBP kinase activity in immunoprecipitates was measured by densitometric scanning of the autoradiogram.

clearly shown that ET-1-induced activation of MBP kinase activities is due to the coordinate activation of the ERK isozymes, ERK1 and ERK2, in rat aortic SMCs.

In the present study we have identified ERK isozymes and demonstrated their activation in response to ET in rat aortic SMCs, indicating that ERKs act as intermediates in signaling pathways of ETRs. The precise role of ERKs in ET action in SMCs must await further studies.

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